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Synthesis of 2- and 17-substituted estrone analogs and their antiproliferative structure–activity relationships compared to 2-methoxyestradiol

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ABSTRACT

A novel series of 17-modified and 2,17-modified analogs of 2-methoxyestradiol (2ME2) were synthesized and characterized. These analogs were designed to retain or potentiate the biological activities of 2ME2 and have diminished metabolic liability. The analogs were evaluated for antiproliferative activity against MDA-MB-231 breast tumor cells, antiangiogenic activity in HUVEC, and estrogenic activity on MCF-7 cell proliferation. Several analogs were evaluated for metabolic stability in human liver microsomes and in vivo in a rat cassette dosing model. This study lead to several 17-modified analogs of 2ME2 that have similar or improved antiproliferative and antiangiogenic activity, lack estrogenic properties and have improved metabolic stability compared to 2ME2.

1. Introduction

Endogenous 2ME2 is produced by hydroxylation of estradiol (E2) at the 2-position and subsequent 2-O-methylation by catechol-*O*-methyltransferase (COMT).[1,2] The apoptotic activity of 2ME2 requires proliferating cells, as it is not cytotoxic to quiescent cell cultures.[1] In numerous tumor models in mice, oral administration of 2ME2 resulted in strong inhibition of tumor growth and tumor neovascularization at doses that produced no apparent signs of toxicity.[3] 2ME2 inhibits several stages of the angiogenic cascade including endothelial cell proliferation, migration, and tubule formation.[1] 2ME2 (Panzem®) has undergone evaluation in several Phase I and Phase II oncology clinical trials[4] and may have a role in other angiogenic diseases such as rheumatoid arthritis.[5]

Orally-delivered steroids such as estradiol and 17-ethynylestradiol are extensively metabolized during uptake from the gastrointestinal tract and by first-pass metabolism in the liver.[6] Two major metabolic pathways that lead to rapid deactivation and excretion of E2 are oxidation by 17-β-hydroxysteroid dehydrogenase of the D-ring 17-hydroxy group to form estrone (E1), and conjugation with sulfate and/or glucuronide of the hydroxyl group at position 3 on the A-ring and at position 17 on the D-ring.[7] Pharmacokinetic (PK) and metabolism data from Phase I studies of 2ME2 in humans and adsorption distribution metabolism and excretion (ADME) studies in rats demonstrated similar metabolic transformations for 2ME2.[4,8,9] As reported for estradiol and 17-ethynyl estradiol, 2ME2 is metabolized principally by oxidative and conjugation processes. These transformations lead to a rapid decrease in the concentration of the active drug.

Several previous studies have been conducted to determine structure–activity relationships (SAR) of 2ME2 analogs.[10] However, the rationale for the exploration of those analogs was to increase the interaction with tubulin, a known target of 2ME2, or to enhance in vitro cytotoxicity, and not to diminish metabolism. The approach described in this paper, based on data from animal models and human clinical PK and metabolism information, was to generate in vitro-active analogs with modifications expected to reduce or prevent metabolism at the 17 position.

In order to block the oxidative and conjugation metabolism discussed above, a series of 2ME2 analogs with polar, ionizable, alkyl, endocyclic, or exocyclic olefins at position 17 were prepared and tested for activity in vitro and for metabolic stability in a variety of models. Additionally, several 17-deoxyestrone analogs were also modified at position 2 as a pilot study to explore the possibility of blocking demethylation of the methoxy group in the A ring. For all these analogs, data are presented on the synthesis and on preliminary biological in vitro screening. To assess the antiangiogenic and antitumor activity of these analogs, inhibition of proliferation was assessed in non-transformed human cells (human umbilical vein endothelial cells, HUVEC) and in three human tumor cell lines (breast carcinoma MDA-MB-231, glioblastoma U87-MG, and prostate carcinoma PC3). Estrogenic activity was assessed by ability to stimulate proliferation of the estrogen-dependent human breast cancer cell line MCF-7. A study was also conducted to determine the metabolic stability of selected analogs toward glucuronidation

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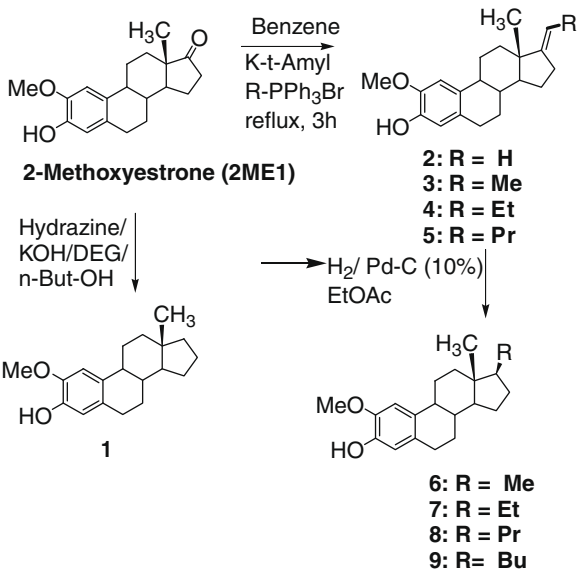
by human liver microsomes, in the presence of the cofactor uridine 5′-diphosphoglucuronic acid (UDPGA). Several lead substituents were also evaluated in a cassette dosing PK study in rats.

2. Chemistry

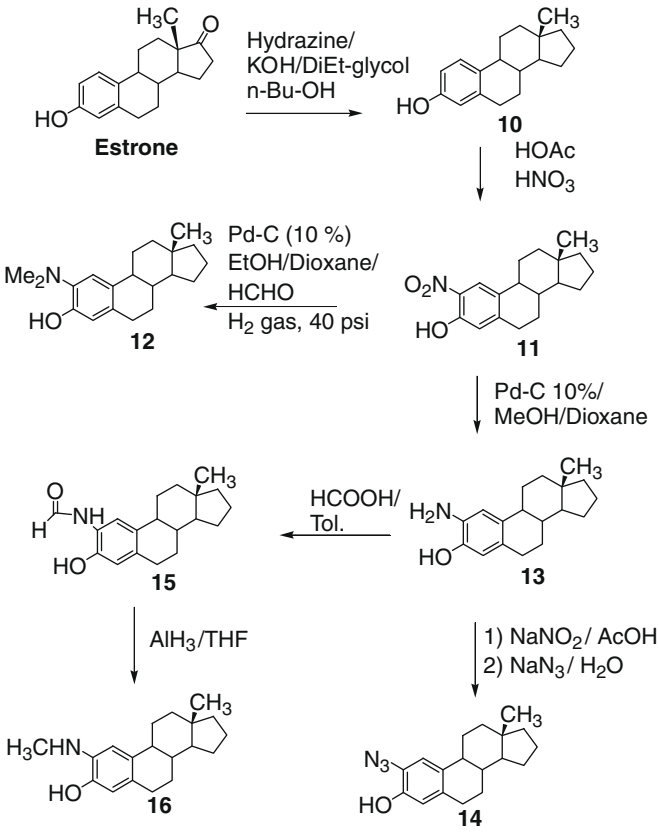
Treatment of 2-methoxyestrone (2ME1) with anhydrous hydrazine in refluxing diethyleneglycol and 1-butanol mixture for 1 h followed by the addition of 3 M equiv of KOH pellets gave 2-methoxy-17-deoxyestrone (**1**) in 90% yield[11] as shown in Scheme 1. 17-Alkyl and alkenyl 2-methoxyestrone analogs were synthesized by treating alkyl triphenylphosphonium bromide with potassium *tert*-amylate in refluxing anhydrous benzene, followed by the addition of 2ME1.[12,13] After work-up, the resulting intermediate was purified on a silica column to give compounds **2–5** (54–84% yield). Hydrogenation of compounds **2–5** in a Parr hydrogenator using Pd/C 10% in ethyl acetate gave 17-β-alkyl analogs **6**, **7**, **8**, and **9** as shown in Scheme 1 (85% yield or greater).

The syntheses of compounds **11**–**16** are shown in Scheme 2. Estrone was reduced to 17-deoxyestrone (**10**) using a similar procedure as shown above for **1**. Reaction of **10** with 1 equiv of HNO_3 in glacial acetic acid for 18 h at room temperature gave compound **11** in 60% yield.[10a] Reduction of **11** in the presence of formaldehyde (37% in H_2O) and Pd/C (10%) in a Parr hydrogenator at 40 psi of hydrogen gas in 1:3 mixture of dioxane/MeOH gave the 2-dimethylamine-17-deoxyestrone **12** in 75% yield.[14] Reduction of **11** without formaldehyde under similar conditions as for **12** gave compound **13** in 90% yield.[10a] Heating **13** in refluxing toluene in the presence of formic acid resulted in the formation of formamide **15** in 75% yield.[15] Reduction of **15** with AlH_3 in THF gave the secondary amine **16** in 70% yield.[16] Reaction of **13** in acetic acid glacial, with sodium nitrite at 0 °C followed by treating with sodium azide in water gave compound **14** in 80% yield.[17]

As shown in Scheme 3, compound **17** was synthesized with 90% yield by heating 2ME1 and *p*-toluenesulfonylhydrazide in refluxing methanol.[18] Similarly the reaction of 2ME1 with hydroxylamine in refluxing ethanol gave compound **21** in 90% yield. Reaction of **17** with n-BuLi in THF gave compound **20** in 80% yield.[19] Compounds **18** and **19** were synthesized by reductive amination of 2ME1 with ammonium acetate and propylamine in the presence of $NaBH_3CN$ in absolute methanol (67–70% yield).[20] Compound **22** was synthesized following the procedure of McGuire,[21] and was then converted to **23** by treating it with $SOCl_2$ at 70 °C followed by NH_3 gas in THF.



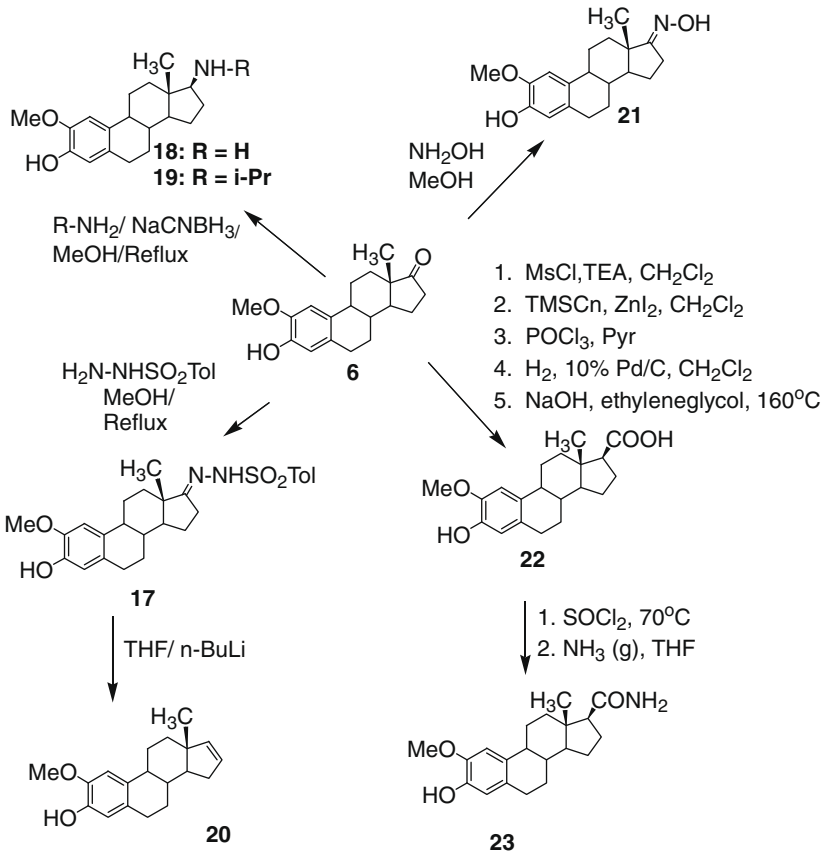
Scheme 1.



Scheme 2.

3. Results and discussion

Before addressing the metabolic stability of the 2ME2 analogs modified at positions 2 and/or 17, an SAR study was conducted in order to select compounds that retain a similar in vitro activity profile to 2ME2. Antiproliferative activity was assessed in endothelial cells (HUVEC) as an in vitro surrogate for antiangiogenic activity, and in three tumor cell lines (MDA-MB-231, U87-MG, and PC3) as a measure of antitumor activity. From the compounds listed in Table 1 only the 17-methylene (**2**), 17-ethylene (**3**), and 16,17-olefin (**20**) exhibited better antiproliferative activities than 2ME2. Increasing carbon chain length at position 17 resulted in a moderate to significant reduction in antiproliferative activity (see compound sub-groups **2** to **5** and **6** to **9**) suggesting that bulkier groups are not well tolerated at that position. We have previously shown a similar limitation on substituent size at the neighboring C-16 position.[10j] Introduction of non-acidic polar groups at position 17 such as amine (**18**), hydroxyl amine (**21**), and carboxyamide (**23**) had little effect on antiproliferative activity compared to 2ME2. However, 17-carboxylic acid (**22**) showed complete loss of activity suggesting that an acidic group is not tolerated at this position. It is also possible that the carboxylic acid prevents the compound from entering the cell due to poor cell permeability. Although slightly less active than 2ME2, the 17-deoxy analog **1** has significantly reduced likelihood of metabolism compared to 2ME2 or several other analogs reported here as it lacks a moiety which could easily be conjugated (e.g., OH, NH_2) or an exocyclic or endocyclic olefin which may be subject to oxidation. D-ring



Scheme 3.

Table 1
Antiproliferative activities of 17-modified analogs of 2ME2

Compound #	R_2	R_{17}	MDA-MB-231 (IC_{50} μM)	U87-MG (IC_{50} μM)	PC3 (IC_{50} μM)	HUVEC (IC_{50} μM)	MC7 SI Relative to 2ME2
2ME2	CH_3O–	OH–	1.00 ± 0.05	8.54 ± 2.29	2.65 ± 0.52	0.84 ± 0.02	1.00 ± 0.05
1	CH_3O–	H	2.20 ± 0.52	7.59 ± 0.21	8.49 ± 2.49	1.32 ± 0.56	0.24 ± 0.06
2	CH_3O–	$=CH_2$	0.24 ± 0.00	N/A	N/A	0.19 ± 0.19	0.89 ± 0.01
3	CH_3O–	$=CHCH_3$	0.58 ± 0.31	N/A	N/A	0.26 ± 0.23	0.89 ± 0.01
4	CH_3O–	$=CHCH_2CH_3$	7.89 ± 2.87	N/A	N/A	3.23 ± 0.63	0.47 ± 0.03
5	CH_3O–	$=CHCH_2CH_2CH_3$	14.00 ± 6.74	N/A	N/A	3.47 ± 0.68	0.51 ± 0.06
6	CH_3O–	CH_3	3.33 ± 0.23	9.39 ± 0.43	6.46 ± 2.31	2.40 ± 0.45	0.89 ± 0.02
7	CH_3O–	$-CH_2CH_3$	48.06 ± 0.91	12.77 ± 0.90	8.70 ± 0.76	3.63 ± 0.12	0.49 ± 0.31
8	CH_3O–	$-CH_2CH_2CH_3$	95.31 ± 3.09	87.88 ± 2.53	85.44 ± 6.05	40.79 ± 1.01	0.69 ± 0.04
9	CH_3O–	$-CH_2CH_2CH_2CH_3$	33.47 ± 3.67	21.11 ± 1.23	28.82 ± 10.2	12.64 ± 6.45	0.59 ± 0.14
17	CH_3O–	$=NNHSO_2Ph\text{-}CH_3$	72.54 ± 13.3	27.32 ± 0.51	27.19 ± 8.91	7.95 ± 2.18	0.43 ± 0.29
18	CH_3O–	NH_2	9.7 7 ± 1.64	19.01 ± 2.07	13.82 ± 7.91	13.37 ± 2.30	0.19 ± 0.09
19	CH_3O–	NH-Pr	17.80 ± 4.06	49.18 ± 4.01	23.40 ± 8.33	18.81 ± 0.48	0.11 ± 0.04
20	CH_3O–	16,17-Olefin	0.73 ± 0.13	0.88 ± 0.07	0.62 ± 0.02	0.71 ± 0.08	0.30 ± .02
21	CH_3O–	=N–OH	8.68 ± 0.75	9.40 ± 0.64	8.11 ± 0.94	4.27 ± 2.11	1.22 ± 0.11
22	CH_3O–	COOH	>100	>100	>100	35.9 ± 2.27	N/A
23	CH_3O–	$CONH_2$	2.51 ± 0.02	11.18 ± 0.04	21.5 ± 3.85	4.55 ± 1.72	N/A

N/A = Not available.
SI = Stimulation index.

olefin compound **20** exhibited slightly better activity than 2ME2 even though it lacks any substituent at the 17 position. This effect may be related to that reported by Rao,[10i] where activity of a small series of 2ME2 analogs modified in the D ring was correlated with the angle of the D-ring to the plane of the ABC ring system.

Previous in vitro metabolism studies indicated that the methoxy moiety of 2ME2 could be oxidatively removed by the action of cytochromes P450 (data not shown). The resultant 2-hydroxyestradiol, if formed in vivo, could be estrogenic. Table 2 shows data from the sub-group of analogs in which we attempted to replace the methoxy group at position 2 by substituents which would not be subject to cytochrome-P450-mediated demethylation. In this subset of molecules with a 17-deoxy moiety, all 2-position substituents tested failed to produce analogs which were equipotent to 2ME2.

2ME2 has low binding affinity for both estrogen receptors α and β, does not engage the estrogen receptor for its antiproliferative activity, nor do estrogen receptor agonists or antagonists influence the activity of 2ME2.[22] Therefore, one goal of this program was to identify analogs that exhibit similar selectivity between estrogenic

Table 2
Antiproliferative activities of 2,17-modified 2ME2 analogs

Compound #	R_2	MDA-MB-231 (IC_{50} μM)	U87-MG (IC_{50} μM)	PC3 (IC_{50} μM)	HUVEC (IC_{50} μM)	MC7 SI Relative to 2ME2
1	CH_3O–	2.20 ± 0.52	7.59 ± 0.21	8.49 ± 2.49	1.32 ± 0.56	0.24 ± 0.06
10	H–	41.26 ± 1.99	22.06 ± 1.64	21.99 ± 2.92	8.22 ± 1.78	1.91 ± 0.31
12HCl	HCl·$(CH_3)_2$N–	39.33 ± 12.6	34.16 ± 6.39	26.00 ± 4.18	7.43 ± 0.96	2.17 ± 0.05
13	H_2N	>100	>100	>80	60.05 ± 0.58	1.49 ± 0.23
14	N3–	7.12 ± 0.24	NT	NT	2.83 ± 0.30	2.04 ± 1.43
15	HCOHN–	24.59 ± 1.32	42.86 ± 3.89	25.99 ± 0.66	6.80 ± 1.74	1.71 ± 0.34
16HCl	HCl·CH_3HN–	26.76 ± 5.31	22.31 ± 1.11	22.82 ± 2.1	4.11 ± 1.51	1.22 ± 0.74

SI = Stimulation index.

and antiproliferative activities as seen for 2ME2 compared to estradiol. The estrogenic activity of 2ME2 and its analogs on estrogen dependent MCF-7 breast cancer cell line was investigated, and activities relative to 2ME2 are presented in the Tables. As shown in Table 1, the only analog exhibiting more estrogenic activity than 2ME2 is compound **21** which contains a highly polar hydroxylamine. All other analogs are further reduced in undesired estrogenic effect compared to 2ME2, and several compounds **1**, **18**, **19**, and **20** exhibited significant reduction in estrogenicity while showing improved antiproliferative properties. The 2- and 17-doubly-modified compounds shown in Table 2 had greater estrogenic and weaker antiproliferative activity compared to 2ME2.

The substituents introduced at the 17 position were chosen to reduce or prevent the facile oxidation or conjugation identified for the 17-hydroxyl moiety of 2ME2. We chose several of the antiproliferative and non-estrogenic analogs for testing in an in vitro metabolism analysis. This study was conducted to determine the metabolic stability of the selected 2ME2 analogs toward glucuronidation in human liver microsomes, in the presence of the cofactor uridine 5′-diphosphoglucuronic acid (UDPGA). Under these conditions it is expected that steroid analogs could be conjugated at either or both positions 3 or 17, as long as there is an availability of appropriate functionality. 2ME2, 17-methylene analog **2**, 17-ethylene analog (**3**), and 17-amino derivative (**18**) were metabolized extensively after 60 min of incubation with human liver microsomes as shown in Table 3 (<10% remaining). The 17-methyl derivative **6** under the same conditions was 47% metabolized in 60 min, whereas analogs **1** (17-deoxy), **7** (17-ethyl), and **15** (17-deoxy, 2-*N*-formamide derivative) were significantly more stable than 2ME2 under these conditions (117%, 72%, and 68% remaining after 60 min, respectively). All analogs tested retain the 3-hydroxyl moiety present in E2 and 2ME2, which is a known target for metabolic conjugation. The variation in stability between analogs such as **1**, **6**, and **7** compared to **2** and **3** suggests that certain D-ring modifications not only prevent conjugation at position 17 but may also exert an effect at the 3 position that can result in decreased conjugation at that site.

Several analogs (**1**, **2**, **3**, **6**, and 2ME2) were tested using rat cassette dosing as a screen for in vivo pharmacokinetic properties. The cassette dosing technique has the advantage of being able to screen several compounds for pharmacokinetic properties simultaneously in a single animal. Plasma values for 2ME2 were below the limit of quantification in this experiment, which can be explained by the low dose used in a cassette dosing experiment. In contrast, all analogs tested showed significant oral bioavailability, with acceptable C_{max}, AUC and half life (Table 4). 17-Ethylene analog **3** had the greatest bioavailability, AUC and C_{max} of the compounds tested. This analog also had a long $t_{1/2}$ (16.4 h). The 17-deoxy analog **1** had the longest $t_{1/2}$ of any of the analogs tested in this experiment (198.9 h). Despite showing acceptable stability in the in vitro microsomal stability study described above, 17-methyl analog **6** had a short $t_{1/2}$ (0.5 h) and had poor bioavailability (9.4%). Based on these cassette dosing data, the leading substituents for position 17 with respect to metabolic stability are 17-deoxy, 17-methylene, and 17-ethylene.

4. Conclusions

A novel series of compounds has been synthesized that retain many of the in vitro biological activities of 2ME2. Analogs lacking the hydroxyl moiety at position 17 cannot be metabolized to 2-methoxyestrone nor conjugated at that position and, in general, they retain antiproliferative activity on both endothelial and tumor cells (Table 1). In vitro metabolic stability studies confirm that several of these analogs are metabolically more stable than 2ME2 (Table 3). Further replacement of the 2-methoxy group of **1** by other moieties (Table 2) such as 2-*N*-formamide (**15**), 2-*N*-methylamino (**16**), or 2-*N*,*N*-dimethylamino (**12**), was intended to prevent metabolic demethylation to potentially estrogenic 2-hydroxyl derivatives. However these substitutions resulted in analogs that exhibited weaker activity profiles and increased estrogenicity, and were therefore not pursued further. The most significant improvement in overall properties came from replacement of the 17-hydroxy group of 2ME2 with a hydrogen or an exocyclic or endocyclic olefin (i.e., **1** (17-deoxy), **2** (17-methylene), and **3** (17-ethylene). These substituents resulted in analogs which showed diminished estrogenic activity, improved metabolic stability in the in vivo rat cassette dosing model, and increased or similar antiproliferative and antiangiogenic activity compared to 2ME2.

Table 3
Metabolic stability analysis of position 17 and 2-substituted 2ME2 analogs

Time (min)	Control (UDPGA)	Control (+BSA,−HLM)	Compound #							
			2ME2 %	**1** %	**2** %	**3** %	**6** %	**7** %	**15** %	**18** %
0	100	100	100	100	100	100	100	100	100	100
15	—	—	58	94	76	75	67	91	101	32
30	—	—	29	101	67	75	57	76	86	30
60	113	93	9.4	117	5.8	0.8	47	72	68	9

Incubations with human liver microsomes (HLM) were performed in 50 mM tris, pH 7.5 containing 10 mM magnesium chloride, 2 mM uridine-5′-diphosphoglucuronic acid (UDPGA), 25 μg alamethicin/mg liver microsomal protein and 5 μM of each test compound. Control incubations containing the specific concentration of substrate and bovine serum albumin (BSA) but lacking human liver microsomes were conducted.
Amount of substrate analog remaining at each timepoint (shown as % remaining relative to 0 min) was determined by LCMS analysis.

Table 4
Rat cassette dosing summary of selected analogs

Compound #	PO C_{max} (nM)	PO $t_{1/2}$ (h)	PO AUC (nM h)	Oral bioavailability (%F)
2ME2	BLOQ	BLOQ	—	—
1	40	198.9	670	44.5
2	40	24.9	572	32.8
3	80	16.4	1163	84.2
6	50	0.5	165	9.4

The in vitro metabolism data indicate that the 3-position in this series of analogs remains a target for conjugation. The lead substituents at position 17 identified here have been combined with modifications at the 3-position to further improve the pharmacokinetic properties, metabolism, and antitumor activity of this series of molecules. The data from these studies are being reported separately.[23]

5. Experimental

Chemicals and reagents of high purity were obtained from Aldrich, Fluka, or Acros chemical companies and were used without further purification. Chemical reactions were monitored by thin layer chromatography using Merck precoated Silica Gel $60F_{254}$ plates. Biotag FLASH-12™ or FLASH-40™ were used for flash chromatography. ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DPX 300 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane as internal standard (0.0 δ). IR spectra were recorded on a Perkin–Elmer 783 spectrometer and wave numbers are reported in cm^{-1}. Melting points were determined on a Kofler apparatus and are uncorrected. Elemental analysis was performed by Atlantic Micro Lab., Norcross, GA, USA.

5.1. Biology

5.1.1. Proliferation assays

MDA-MB-231 human breast carcinoma cells, U87-MG human glioblastoma cells and PC3 human prostate carcinoma cells were grown in DMEM containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan UT) supplemented with 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Irvine Scientific, Santa Anna, CA). Proliferation was assessed by detection of DNA synthesis by use of the 5-bromo-2′-deoxyuridine (BrdU) cell proliferation colorimetric ELISA kit from Roche (Indianapolis, IN) according to the manufacturer's instructions. For BrdU assays, the cells were seeded at 2000 cells/well in a 96-well plate, allowed to attach overnight and then exposed to increasing concentrations of the individual compounds for 48 h. Each condition was assessed in triplicate and the experiments were carried out a minimum of two times. Results in Table 1 are means of the data from each independent experiment ± SD.

HUVEC were grown in EGM (Clonetics). HUVEC were seeded at 5000 cells/well in 96-well plates. After being allowed to attach overnight, the cells were washed with PBS and incubated in the absence of growth factor for 24 h (EBM, 2% FBS, Clonetics). Cells were then treated with increasing concentrations of drug in EBM containing 2% FBS and 10 ng/mL bFGF for 48 h at 37 °C. The preparation of the drugs and BrdU proliferation assay were performed as indicated above.

MCF-7, an estrogen dependent breast carcinoma cell line, was maintained in DMEM/F12 (1:1) containing 10% (v/v) FBS (Hyclone Laboratories, Logan, UT) and 1X antibiotic–antimytotic. MCF-7 cells, used between passage 60 and passage 90, were the kind gift of Dr. Dorraya El Ashry, of Georgetown University. For MCF-7 estrogen-dependent proliferation assays the cells were seeded in complete media at 20–30,000 cells/well in 24-well plates. After allowing the cells to adhere overnight the seeding density was determined by cell count. Cells were then washed with PBS (37 °C) and starved by placing them in IMEM–phenol red free media containing 2% charcoal–dextran fetal bovine–stripped serum (Georgetown University) and 1X antibiotic–antimitotic. After 3 days of starvation, cells were treated with or without increasing concentrations of each compound, replacing the media every 2–3 days, and counted after 8 or 10 days of treatment. Proliferation was measured by cell counting using a Coulter Z1 cell counter (Coulter Corporation, Hialeah, FL). Each condition was done in triplicate in at least 2 independent experiments. Results are presented as the stimulation index (SI) relative to 2ME2 (defined as 1.00).

Ex vivo liver microsome studies were performed at BD Gentest in Woburn MA. The metabolic study with human liver microsomes were performed in 50 mM tris, pH 7.5 containing 10 mM magnesium chloride, 2 mM uridine-5′-diphosphoglucuronic acid (UDPGA), 25 μg alamethicin/mg liver microsomal protein, and 5 μM of each test compound. Control incubations containing the specific concentration of substrate and bovine serum albumin (BSA) but lacking human liver microsomes were conducted. For control samples lacking cofactor UDPGA, incubation were performed in 50 mM tris pH 7.5 containing 10 mM magnesium chloride, 25 μg alamethicin/mg liver microsomal protein and 5 μM of each test compound. After incubation times of 0, 15, 30 and 60 min, an aliquot was removed and added to 1.5 × vol/vol acetonitrile containing internal standard. The samples were frozen at −20 °C for subsequent analysis. LCMS analysis was performed using Waters C18 Symmetry 2.1 mm × 50 mm column eluted with water (A)/methanol (B) using gradient from 50% B to 100% B over 5 min, 100% B to 7 min, 50% B to 8.5 min and 50% B to 10 min at flow rate of 0.2 mL/min. Mass spectral data was recorded in the negative ion mode.

The cassette dosing experiment was conducted by Ricerca Biosciences (Concord OH) using a standard protocol. Six Sprague-Dawley rats were implanted with jugular vein canulae. The compounds (**3**–**5**/cassette) were administered to three rats intravenously and three rats orally. Blood was taken at 10 timepoints for intravenous and eight time points for the oral route. Compounds were extracted by plasma protein precipitation and analyzed by LCMSMS. Quantification was carried out against a standard curve prepared by spiking known amounts of the same mixture of compounds into blank rat plasma. PK parameters were calculated using WinNonlin (Pharsight Inc.).

5.2. Chemistry

5.2.1. Synthesis of 2-methoxyestra-1,3,5(10)-triene-3-ol (1)

Into a stirring suspension of 2-methoxyestrone (9.0 g, 30 mmol) in 60 mL diethyleneglycol, 20 mL 1-butanol and 2 mL anhydrous hydrazine (60 mmol) was added. The reaction mixture was heated under reflux for 1 h until the solution cleared. After the mixture cooled to 50 °C, KOH pellets (5.04 g, 90 mmol) was added and the butanol was distilled. The reaction mixture was heated at 50 °C for 2 h and then cooled to room temperature. After pouring into ice (50 g), 20 mL 6 N HCl was added and the reaction mixture stirred to give a white solid product. The product was separated by filtration, washed with cold water and dried under vacuum to give 7.5 g (80%) of product. The product was purified on a silica gel column eluted with $CHCl_3$. Mp 111–112 °C; ^1H NMR ($CDCl_3$) δ, 6.93 (1H, s, aromatic), 6.75 (1H, s, aromatic), 5.35 (1H, s, phenol), 3.95 (3H, s, methoxy), 2.95 (2H, dd, J = 5.0, 3.0 Hz), 2.25 (2H, m), 1.95 (2H, m), 1.95–1.05 (11H, m), 0.8 (3H, s). Anal. Calcd for $C_{19}H_{26}O_2$: C, 79.68; H, 9.15. Found: C, 79.79; H, 9.03.

5.2.2. Synthesis of 17(20)-methyleneestra-1,3,5(10)-triene-3-ol (2). (General procedure A)

Potassium-*tert*-amylate in 4.35 mL toluene (1.54 M, 6.69 mmol, prepared as reported by Schow et al.[13]) was added to a suspension of methyl triphenylphosphonium bromide (2.39 g, 6.69 mmol) in anhydrous benzene (50 mL) and refluxed for 30 min. 2-Methoxyestrone (300 mg, 1 mmol) in warm benzene (5 mL) was then added and the mixture was refluxed for 3 h. The reaction was cooled to room temperature, poured into 100 mL water and washed with ether (2 × 100 mL). The combined organics were washed with 6 M HCl (1 × 100 mL), saturated $NaHCO_3$ (1 × 100 mL), water (1 × 100 mL), and brine (1 × 100 mL), then dried with sodium sulfate, filtered, and concentrated via rotary evaporation to give a semi-solid yellowish oil. The crude product was purified by silica gel column chromatography using 95:5 hexane/ethyl acetate as an eluent to yield 220 mg (73% yield) of white solid as 17(20)-methyleneestra-1,3,5(10)-triene-3-ol (**2**). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.83 (s, 1H), 6.67 (s, 1H), 5.44 (br s, 1H), 4.70 (t, J = 2.26 Hz, 2H), 3.89 (s, 3H), 2.86–2.74 (m, 2H), 2.64–2.49 (m, 1H), 2.39–2.17 (m, 3H), 2.02–1.78 (m, 3H), 1.65–1.19 (m, 4H), 0.85 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.2, 144.9, 143.8, 132.3, 130.0, 115.0, 108.5, 101.2, 77.6, 56.5, 53.9, 44.7, 39.2, 36.2, 29.9, 29.5, 28.1, 27.3, 24.3, 19.0. Anal. Calcd for $C_{20}H_{26}O_2$: C, 80.48; H, 8.79. Found: C, 80.60; H, 8.77.

5.2.3. Synthesis of 2-methoxy-19(*E*/*Z*)-norpregna-1,3,5(10)17(20)-tetraene-3-ol (3)

Reaction conditions as above except reaction scale was doubled and ethyl triphenylphosphonium bromide was used, from 2-methoxyestrone (613 mg, 2.04 mmol) obtain 540 mg (1.73 mmol, 84% yield) of final product. Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.82 (s, 1H), 6.67 (s, 1H), 5.44 (s, 1H), 5.23–5.07 (m, 1H), 3.88 (s, 3H), 2.86–2.72 (m, 2H), 2.51–2.38 (m, 2H), 2.38–2.17 (m, 3H), 1.99–1.88 (m, 1H), 1.83–1.68 (m, 4H), 1.49–1.20 (m, 5H), 0.94 (s, *Z* isomer) and 0.80 (s, *E* isomer, total 3H, ratio 5:1, respectively). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.0 (*E* isomer) and 150.7 (*Z* isomer), 145.0, 143.8, 132.4, 130.0, 115.0, 113.8, 110.6 (*Z* isomer) and 108.4 (*E* isomer), 56.5, 55.6, 54.1, 45.0 (*Z* isomer) and 44.5 (*E* isomer), 39.0 (*E* isomer) and 38.7 (*Z* isomer), 37.7 (*Z* isomer) and 36.6 (*E* isomer), 31.9, 29.5, 28.1 (*E* isomer) and 28.0 (*Z* isomer), 27.7 (*Z* isomer) and 27.4 (*E* isomer), 24.5 (*Z* isomer) and 24.4 (*E* isomer), 19.5 (*E* isomer) and 17.4 (*Z* isomer), 14.0 (*E* isomer) and 13.6 (*Z* isomer). Anal. Calcd for $C_{21}H_{28}O_2$: C, 80.73; H, 8.79. Found: C, 80.60; H, 8.77.

5.2.4. Synthesis of 2-methoxy-17(20)-*E*/*Z*-propylideneestra-1,3,5(10)-triene-3-ol (4)

Reaction conditions as general procedure A except reaction scale was doubled and propyl triphenylphosphonium bromide was used, from 2-methoxyestrone (614.2 mg, 2.04 mmol) obtain 358.9 mg (1.10 mmol, 54% yield) of final product. Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.66 (s, 1H), 5.44 (s, 1H), 5.07 (t, J = 7.4 Hz, 1H), 3.88 (s, 3H), 2.88–2.71 (m, 2H), 2.53–2.04 (m, 6H), 2.00–1.87 (m, 1H), 1.76 (t, J = 9.9, 12 Hz, 2H), 1.49–1.18 (m, 6H), 0.99 (t, J = 7.5 Hz, 3H), 0.94 (s, *Z* isomer) and 0.81 (s, *E* isomer, total 3H, *Z*/*E* ratio 5:1, respectively). ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.5 (*E* isomer) and 149.2 (*Z* isomer), 145.0,143.8, 132.4, 130.0, 122.3 (*Z* isomer) and 118.6 (*E* isomer), 115.0, 108.4, 56.5, 55.7 (*Z* isomer) and 54.0 (*E* isomer), 45.0 (*Z* isomer) and 44.9 (*E* isomer), 44.5 (*Z* isomer) and 44.2 (*E* isomer), 39.1 (*E* isomer) and 38.8 (*Z* isomer), 37.7 (*Z* isomer) and 36.6 (*E* isomer), 31.9, 29.5, 28.2 (*E* isomer) and 28.0 (*Z* isomer), 27.7 (*Z* isomer) and 27.4 (*E* isomer), 26.7 (*E* isomer) and 24.5 (*Z* isomer), 22.2 (*E* isomer) and 21.3 (*Z* isomer), 19.5 (*E* isomer) and 17.9 (*Z* isomer), 15.8 (*Z* isomer) and 14.7 (*E* isomer). Anal. Calcd for $C_{22}H_{30}O_2$: C, 80.94; H, 9.26. Found: C, 80.71; H, 9.30.

5.2.5. Preparation of 2-methoxy-17(20)-(*E*/*Z*)-butylideneestra-1,3,5(10)-triene-3-ol (5)

Reaction conditions as above in general procedure A except reaction scale was doubled and butyl triphenylphosphonium bromide was used, from 2-methoxyestrone (593.6 mg, 1.97 mmol) obtain 532.1 mg (1.56 mmol, 79% yield) of final product. Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.82 (s, 1H), 6.66 (s, 1H), 5.43 (s, 1H), 5.08 (t, J = 7.4 Hz, 1H), 3.88 (s, 3H), 2.81–2.77 (m, 2H), 2.54–2.01 (m, 7H), 1.99–1.87 (m, 1H), 1.76 (app t, J = 9.6, 12.6 Hz, 2H), 1.49–1.26 (m, 6H), 0.97–0.89 (m, *Z* isomer, terminal butyl methyl) and 0.80 (s, *E* isomer, total 6H, *Z*/*E* ratio 9:1, respectively by difference in integration). ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.2 (*E* isomer) and 149.8 (*Z* isomer), 145.0, 143.8, 132.4, 130.0, 120.5 (*Z* isomer) and 116.7 (*E* isomer), 115.0, 108.4, 56.5, 55.7 (*Z* isomer) and 54.1 (*E* isomer), 44.9, 44.5 (*Z* isomer) and 44.3 (*E* isomer), 39.1 (*E* isomer) and 38.8 (*Z* isomer), 37.8 (*Z* isomer) and 36.6 (*E* isomer), 32.0, 31.0 (*E* isomer) and 30.1 (*Z* isomer), 29.5, 28.2 (*E* isomer) and 28.0 (*Z* isomer), 27.7 (*Z* isomer) and 27.4 (*E* isomer), 24.5, 24.3 (*Z* isomer) and 23.3 (*E* isomer), 19.6 (*E* isomer) and 17.9 (*Z* isomer), 14.4. Anal. Calcd for $C_{23}H_{32}O_2$: C, 81.13; H, 9.47. Found: C, 81.32; H, 9.55.

5.2.6. Synthesis of 2-methoxy-17-β-methylestra-1,3,5(10)-triene-3-ol (6). (General procedure B)

Compound **2** (471.9 mg, 1.58 mmol) was dissolved in ethyl acetate (20 mL), flushed with argon, Pd/C 10% (47.5 mg) was added and the reaction mixture was then subjected to hydrogenation in a Parr hydrogenator for 1 h under 30 psi of hydrogen. The reaction mixture was then filtered through a Celite pad and solvent was removed to yield 472.5 mg white crystals (97% yield) of the final product. Mp 133–134 °C; ^{1}H NMR ($CDCl_3$) δ, 6.82 (s, 1H), 6.66 (s, 1H), 5.43 (s, 1H), 3.88 (s, 3H), 2.85–2.70 (m, 2H), 2.32–2.15 (m, 2H), 1.94–1.68 (m, 4H), 1.52–1.12 (m, 8H), 0.90 (d, J = 6.9 Hz, 3H), 0.61 (s, 3H). ^{13}C NMR ($CDCl_3$) δ, 144.90, 143.75, 132.65, 130.11, 114.99, 108.51, 56.46, 55.21, 45.58, 44.85, 42.74, 39.39, 37.97, 30.65, 29.52, 28.38, 27.21, 24.83, 14.34, 12.44. Anal. Calcd for $C_{20}H_{28}O_2$: C, 79.96; H, 9.39. Found: C, 79.98; H, 9.49.

5.2.7. Synthesis of 2-methoxy-17-β-ethyl-estra-1,3,5(10)-triene-3-ol (7)

Compound **7** was synthesized according to the general procedure B above for **6** except compound **3** was used for hydrogenation to give 91% reaction yield. Mp 118–119 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.82 (s, 1H), 6.66 (s, 1H), 5.43 (s, 1H), 3.88 (s, 1H), 2.78 (app t, J = 10.2, 4.5 Hz, 3H), 2.30–2.15 (m, 2H), 2.00–1.82 (m, 3H), 1.79–1.69 (m, 1H), 1.62–1.08 (m, 11H), 0.92 (app t, J = 7.5, 6.6 Hz, 3H), 0.63 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 144.90, 143.74, 132.66, 130.10, 114.98, 108.49, 56.45, 55.36, 53.58, 44.91, 42.85, 39.19, 38.47, 29.51, 28.65, 28.35, 27.24, 24.67, 23.57, 13.78, 12.95. Anal. Calcd for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62. Found: C, 79.95l; H, 9.71.

5.2.8. Synthesis of 2-methoxy-17-β-propylestra-1,3,5(10)-triene-3-ol (8)

Compound **8** was synthesized according to the general procedure B above for compound **6** except compound **4** was used to give 95% yield. Mp 124–126 °C; ^{1}H NMR ($CDCl_3$) δ, 6.82 (s, 1H), 6.66 (s, 1H), 5.43 (s, 1H), 3.88 (s, 3H), 2.84–2.70 (m, 2H), 2.31–2.16 (m, 2H), 1.97–1.67 (m, 4H), 1.52–1.06 (m, 12H), 0.93 (t, J = 6.7 Hz, 3H), 0.63 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 144.91, 143.75, 132.66, 130.10, 115.00, 108.50, 56.46, 55.31, 51.34, 44.92, 42.83, 39.21, 38.41, 33.15, 29.52, 28.98, 28.37, 27.24, 24.77, 22.39, 15.06, 12.97. Anal. Calcd for $C_{22}H_{32}O_2$: C, 80.44; H, 9.82. Found: C, 80.20; H, 9.86.

5.2.9. Synthesis of 2-methoxy-17-β-butylestra-1,3,5(10)-triene-3-ol (9)

Compound **9** was synthesized according to the general procedure B above for compound **6** except compound **5** was used to give 85% yield. Mp 119–120 °C; ^{1}H NMR ($CDCl_3$) δ, 6.82 (s, 1H), 6.66 (s, 1H), 5.43 (s, 1H), 3.89 (s, 3H), 2.78 (app t, J = 10.8, 4.5 Hz, 2H), 2.30–2.16 (m, 2H), 1.96–1.83 (m, 3H), 1.79–1.69 (m, 1H), 1.51–1.07 (m, 15H), 0.92 (t, J = 9, 6.9 Hz, 4H), 0.63 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 144.90, 143.74, 132.66, 130.09, 114.99, 108.48, 56.46, 55.31, 51.52, 44.92, 42.84, 39.20, 38.42, 31.56, 30.49, 29.52, 29.02, 28.36, 27.23, 24.75, 23.63, 14.60, 12.96. Anal. Calcd for $C_{23}H_{34}O_2$: C, 80.65; H, 10.01. Found: C, 80.90; H, 10.10.

5.2.10. Synthesis of estra-1,3,5(10)-triene-3-ol (10)

Into a stirring suspension of estrone (8.1 g, 30 mmol) in 60 mL diethylene glycol, 20 mL 1-butanol and 2 mL hydrazine (anhydrous) (60 mmol) were added. The reaction mixture was heated under reflux for 1 h until the solution cleared. After cooling the reaction mixture to 50 °C, KOH pellets (5.04 g, 90 mmol) was added and butanol was distilled. The reaction mixture was heated at 50 °C for 2 h and then cooled to room temperature. The reaction mixture was poured onto ice (50 g), and then 20 mL 6 N HCl was added with stirring to give a white solid product. The product was isolated by filtration, washed with cold water and dried under vacuum to give 7.5 g (90%) product. The product was purified on a silica gel column eluted with $CHCl_3$/MeOH 99:1. Mp 136–137 °C; ^{1}H NMR (**6**) ($CDCl_3$,) δ, 7.18 (1H, d, J = 8.0 Hz, aromatic), 6.64 (1H, d, J = 8.0 Hz, aromatic), 6.58 (1H, s, aromatic), 4.58 (1H, s, phenol), 2.85 (2H, dd, J = 5.0, 3.0 Hz), 2.25 (2H, m), 1.95 (2H, m), 1.80–1.05 (11H, m), 0.85 (3H, s). Anal. Calcd for $C_{18}H_{24}O$: C, 84.32; H, 9.44. Found: C, 84.01; H, 9.67.

5.2.11. Synthesis of 2-nitroestra-1,3,5(10)-triene-3-ol (11)

Into a solution of 17-deoxyestrone (**6**), (760 mg, 3 mmol) in 20 mL acetic acid glacial, 0.19 mL (3 mmol) of concd HNO_3 was added at 2 °C. The reaction mixture was brought to room temperature and stirred for 18 h. The reaction mixture was diluted with water and products were extracted with ethyl ether. The ether layer was washed with water and brine and dried over $CaSO_4$ (anhydrous), filtered and evaporated to give a yellow solid. The product was purified on flash silica gel column eluted with hexane/CH_2Cl_2 4:1 mixture to give a yellow crystal product (400 mg, 55%). Mp = 118–119 °C; ^{1}H NMR (**7**) ($CDCl_3$,) δ, 10.42 (1H, s, phenol), 8.05 (1H, s, aromatic), 6.84 (1H, s, aromatic), 2.95 (2H, dd, J = 5.0, 3.0 Hz), 2.30 (2H, m), 1.95 (2H, m), 1.80–1.15 (11H, m), 0.80 (3H, s). Anal. Calcd for $C_{18}H_{23}NO_3$: C, 71.73; H, 7.69; N, 4.65. Found: C, 71.69; H, 7.64; N, 4.39.

5.2.12. Synthesis of 2-*N*,*N*-dimethylaminoestra-1,3,5(10)-triene-3-ol (12)

Compound **7** (301 mg, 1.0 mmol) was dissolved in 30 mL dioxane/methanol 1:3 mixture and was hydrogenated in a Parr hydrogenator at 40 psi of hydrogen gas in the presence of 0.4 mL formaldehyde (37%) and Pd/C 10% (200 mg) for 4 h. After filtering the reaction mixture through a Celite filter pad the solvents were evaporated under vacuum to give a white powder (240 mg, 95%). ^{1}H NMR ($CDCl_3$,) δ 7.15 (1H, s, aromatic), 6.74 (1H, s, aromatic), 2.85 (2H, dd, J = 5.0, 3.0 Hz), 2.65 (6H, s, dimethylamino) 2.25 (2H, m), 1.95 (2H, m), 1.75–1.05 (11H, m), 0.80 (3H, s). Anal. Calcd for $C_{20}H_{29}NO$: C, 80.22; H, 9.76; N, 4.68. Found: C, 80.07; H, 10.13; N, 4.25.

5.2.13. Synthesis of 2-aminoestra-1,3,5(10)-triene-3-ol (13)

Compound **7** (170 mg, 0.5 mmol) was dissolved in 30 mL dioxane/methanol 1:5 mixture and hydrogenated in a Parr hydrogenator at 40 psi of hydrogen in the presence of Pd/C 10% (200 mg) for 4 h. After filtering the reaction mixture through Celite filtering agent, the solvents were evaporated under vacuum to give white crystals (140 mg, 95%). Mp 222–223 °C; ^{1}H NMR ($CDCl_3$,) δ, 6.75 (1H, s, aromatic), 6.44 (1H, s, aromatic), 3.80 (2H, br, amine) 2.75 (2H, dd, J = 5.0, 3.0 Hz), 2.20 (2H, m), 1.95 (2H, m), 1.75–1.05 (11H, m), 0.80 (3H, s). Anal. Calcd for $C_{18}H_{25}NO$: C, 79.66; H, 9.28; N, 5.16. Found: C, 79.96; H, 8.78; N, 5.14.

5.2.14. Synthesis of 2-azido-estra-1,3,5(10)-triene-3-ol (14)

Into a solution of 2-amino-estradiol (144 mg, 0.5 mmol) in 3 mL acetic acid glacial, a solution of sodium nitrite (48 mg, 0.7 mmol) in 1 mL water was added at 0 °C. The color of the reaction mixture changed to orange-yellow. After stirring at 0 °C for 30 min a solution of sodium azide (45 mg, 0.7 mmol) in water was added. The color of the reaction mixture changed to orange-red. Temperature was maintained at 0 °C for 30 min and then raised to room temperature. After stirring for 1 h, the solvents were evaporated under vacuum and the remaining solid was dissolved in chloroform. The chloroform layer was washed with water and brine and dried over $CaSO_4$ anhydrous, filtered and evaporated to give a light brown foamy solid. The product was purified by flash silica gel column eluted with $CHCl_3$/MeOH 99:1 mixture to give 100 mg light yellow foamy solid (70%). IR and ^{1}H NMR in $CDCl_3$ confirmed the product as 2-azido-estra-1,3,5(10)-triene-3-ol. ^{1}H NMR ($CDCl_3$) δ, 7.03 (1H, s, aromatic), 6.65 (1H, s, aromatic), 5.25 (1H, s, phenol), 2.85 (2H, dd, J = 5.0, 3.0 Hz), 2.25 (2H, m), 1.95 (2H, m), 1.95–1.05 (11H, m), 0.78 (3H, s). Anal. Calcd for $C_{18}H_{23}N_3O$: C, 72.70; H, 7.80; N, 14.13. Found: C, 72.87; H, 7.91; N, 14.33.

5.2.15. Synthesis of 2-*N*-formamidoestra-1,3,5(10)-triene-3-ol (15)

Formic acid (0.2 mL) was added into a hot solution (60 °C) of **9** (135 mg, 0.5 mmol) in 5 mL toluene. The toluene and water mixture was azeotroped for 1 h. When 1/3 of the toluene was distilled the reaction mixture was cooled to room temperature to give white crystals of 2-*N*-formamido-17-deoxyestrone (100 mg, 75%). Mp 190–191 °C; ^{1}H NMR ($CDCl_3$,) δ, 8.2 (1H, s), 7.6 (1H, br), 7.05 (1H, s, aromatic), 6.84 (1H, s, aromatic), 2.90 (2H, dd, J = 5.0, 3.0 Hz), 2.20 (2H, m), 1.85 (2H, m), 1.8–1.15 (11H, m), 0.75 (3H, s). Anal. Calcd for $C_{19}H_{25}NO_2$: C, 76.22; H, 8.42; N, 4.68. Found: C, 76.28; H, 8.72; N, 4.52.

5.2.16. Synthesis of 2-*N*-methylaminoestra-1,3,5(10)-triene-3-ol (16)

Into solution of AlH_3 (5 mmol) (formed in situ) in 15 mL THF (anhydrous), amide **10** (150 mg, 0.5 mmol) in 5 mL THF was added at room temperature and stirred for 2 h. THF/H_2O (0.4 mL, 1:1) was added dropwise, followed by 1.5 mL NaOH (15% solution). This suspension was stirred for 20 min after which, ether was added (20 mL). The $Al(OH)_3$ precipitates were removed by filtration. Solvents were evaporated and the product was purified on a flash silica gel column eluted with a hexane/ether 1:1 mixture to give a white solid product (120 mg, 70%). The HCl salt of the product was formed by dissolving **11** in IPA/HCl (gas) solution. The salt was precipitated by adding ethyl ether, which was isolated by filtration, washed by ether and dried under vacuum. Mp 266–267 °C (HCl salt, decompose); ^{1}H NMR (HCl salt) (DMSO-d_6,) δ, 10.25 (1H, s), 10.16 (1H, br), 7.15 (1H, s, aromatic), 6.84 (1H, s, aromatic), 2.85 (3H, s, methylamine), 2.75 (2H, dd, J = 5.0, 3.0 Hz), 2.20 (2H, m), 1.95 (2H, m), 1.75–1.00 (11H, m), 0.70 (3H, s). Anal. Calcd for $C_{19}H_{27}NO$: C, 79.95; H, 9.53; N, 4.91. Found: C, 80.06; H, 9.78; N, 5.13.

5.2.17. Synthesis of 2-methoxyestra-1,3,5(10)-triene-17-*p*-toluenesulfonhydrazone-3-ol (17)

To a solution of 2-methoxyestrone (0.60 g, 2 mmol) in 2 mL methanol, *p*-toluene-sulfonylhydrazide (0.46 g, 2.5 mmol) was

added. The reaction mixture was heated under reflux for 1 h. The methanol was evaporated and product was crystallized from ethyl ether to give 720 mg (80%) white solid. ^{1}H NMR ($CDCl_3$,) δ, 7.90 (2H, d, J = 7.3, aromatic), 7.37 (2H, d, J = 7.35, aromatic), 6.95 (1H, s), 6.70 (1H, s, aromatic), 6.63 (1H, s, aromatic), 5.50 (1H, br), 3.95 (3H, s, methoxy), 2.85 (3H, s), 2.89 (2H, dd, J = 5.0, 3.0 Hz), 2.55 (2H, m), 2.30 (2H, m), 2.10–1.35 (11H, m), 0.95 (3H, s). Anal. Calcd for $C_{26}H_{32}N_2O_4S$: C, 66.64; H, 6.88; N, 5.98. Found: C, 66.61; H, 6.80; N, 6.01.

5.2.18. Synthesis of 2-methoxyestra-1,3,5(10)-triene-17-amino-3-ol (18)

A solution of 2-methoxyestrone (300 mg, 1 mmol), ammonium acetate (0.77 g, 10 mmol) and $NaBH_3CN$ (33 mg, 7 mmol) in 10 mL absolute methanol was stirred for 48 h at 25 °C. After adding 0.5 mL concentrated HCl the methanol was evaporated. After diluting the reaction mixture with 10 mL water, it was basified with 1 N NaOH until pH >10 and the product was extracted with 2 × 20 mL portions of ethyl acetate. The combined extracts were dried over $MgSO_4$ (anhydrous), filtered and evaporated to give 200 mg of white solid (67%). Product was recrystallized from the isopropanol/hexane mixture. ^{1}H NMR ($CDCl_3$) δ, 6.93 (1H, s, aromatic), 6.69 (1H, s, aromatic), 5.50 (1H, br), 3.91 (3H, s, methoxy), 2.85 (2H, dd, J = 5.0, 3.0 Hz), 2.55 (2H, m), 2.30 (2H, m), 2.10–1.35 (11H, m), 1.03 (3H, s). Anal. Calcd for $C_{19}H_{27}NO_2$: C, 75.71; H, 9.03; N, 4.65. Found: C, 75.74; H, 8.83; N, 4.35.

5.2.19. Synthesis of 2-methoxyestra-1,3,5(10)-triene-17-propylamino-3-ol (19)

To a solution of 2-methoxyestrone (300 mg, 1 mmol) in 10 mL absolute methanol was added propylamine (0.49 mL, 6 mmol), 0.4 mL 5 N HCl/MeOH and $LiBH_3CN$ (33 mg, 7 mmol) and stirred for 60 h at 25 °C. The methanol was evaporated and after diluting the reaction mixture with 10 mL water, it was basified with 1 N NaOH until pH >10 and product was extracted with 2 × 20 mL portions of ethyl acetate. The combined extracts were dried over $MgSO_4$ (anhydrous), filtered and evaporated to give 220 mg of white solid (70%). Product was recrystallized from an isopropanol/hexane mixture, mp 233.5–234.5 °C. ^{1}H NMR ($CDCl_3$) δ, 7.03 (1H, s, aromatic), 6.79 (1H, s, aromatic), 5.70 (1H, br), 3.93 (3H, s, methoxy), 3.10–2.91 (3H, m), 2.85 (2H, dd, J = 5.0, 3.0 Hz), 2.55 (2H, m), 2.30 (2H, m), 2.10–1.35 (14H, m), 1.03 (3H, s), 0.93 (3H, t, J = 5.5 Hz). Anal. Calcd for $C_{22}H_{33}NO_2$: C, 76.92; H, 9.68; N, 4.08. Found: C, 77.12; H, 9.60; N, 3.99.

5.2.20. Synthesis of 2-methoxy-1,3,5(10)16-estratetraene-3-ol (20)

2-Methoxyestrone-*p*-tosylhydrazone (**17**) (1.5757 g, 3.3 mmol) was dissolved in anhydrous tetrahydrofuran (50 mL) in a flame dried 250 mL round bottomed flask and cooled to −10 °C. The mixture was stirred and *n*-butyl lithium (5.3 mL, 2.5 M in hexanes, Aldrich) was added dropwise. The mixture was warmed to room temperature over 6 h. Ice (~5 g) was added, followed by saturated ammonium chloride (~50 mL). The mixture was transferred to 250 mL separatory funnel, shaken, and separated. The aqueous layer was washed with ethyl acetate (50 mL) and the organics were combined. The organics were washed with 1 N HCl (20 mL) and brine (50 mL), then dried with magnesium sulfate, filtered, and solvent was removed under reduced pressure. The resulting solid was dissolved in acetone (~10 mL) and solvent was removed under reduced pressure two times. Product was purified by silica gel column eluted with hexanes/$CHCl_3$ 1:1 mixture to obtain crystalline solid (745 mg, 80% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.68 (s, 1H), 5.97–5.91 (m, 1H), 5.80–5.74 (m, 1H), 5.43 (s, 1H), 3.88 (s, 3H), 2.90–2.71 (m, 2H), 2.38–1.34 (m, 11H), 0.82 (s, 3H). Anal. Calcd for $C_{19}H_{24}O_2$: C, 80.24; H, 8.50. Found: C, 80.12; H, 8.60.

5.2.21. Synthesis of 2-methoxyestra-1,3,5(10)-triene-17-one-oxime-3-ol (21)

To a solution of 2-methoxyestrone (0.90 g, 3 mmol) in 10 mL methanol, sodium acetate (anhydrous) (3 g, 30 mmol), and hydroxylamine hydrochloride (2.1 g in 3 mL water) were added. The reaction mixture was heated under reflux for 3 h to give a clear solution. The solvents were evaporated and the product was extracted with chloroform. The chloroform layer was washed with water (20 mL), brine and dried over $CaSO_4$ (anhydrous), filtered and evaporated to give 820 mg (90%) of white solid. Mp 208–209 °C; ^{1}H NMR ($CDCl_3$,) δ, 6.90 (1H, s, aromatic), 6.75 (1H, s, aromatic), 5.55 (1H, br), 3.90 (3H, s, methoxy), 2.85 (2H, dd, J = 5.0, 3.0 Hz), 2.55 (2H, m), 2.30 (2H, m), 2.10–1.35 (11H, m), 1.03 (3H, s). Anal. Calcd for $C_{19}H_{25}NO_3$: C, 72.35; H, 7.99; N, 4.44. Found: C, 72.39; H, 7.91; N, 4.43.

5.2.22. Synthesis of 3-hydroxy-2-methoxy-estra-1,3,5(10)-triene-17α/β-carboxylic acid (22)

A slurry of steroid (3-hydroxy-2-methoxy-estra-1,3,5(10)-triene-17 cyano) (590 mg, 1.5 mmol) in ethylene glycol (22 mL) was added to a solution of NaOH (1.9 g) in ethylene glycol (13 mL) and refluxed for 4 h. The reaction was cooled to room temperature, water was added, and solution was acidified to pH 1 with HCl. A precipitate started to form, and the mixture was placed in fridge overnight. Crystals were isolated and purified by chromatography with 9:1:0.1 $CHCl_3$/MeOH/NH_4OH. Yield: 50%, 250 mg, 0.76 mmol. ^{1}H NMR ($CDCl_3$) δ, 6.72 (s, beta isomer) and 6.69 (s, alpha isomer, total 1H, ratio 2:1, respectively), 6.57 (s, 1H), 5.38 (s, br, 1H), 3.79 (s, beta isomer) and 3.77 (s, alpha isomer, total 3H, ratio 2:1, respectively), 2.75–2.66 (m, 2H), 2.58 (dd, J = 7.9, 3.4 Hz, alpha isomer) and 2.42 (app t, J = 9.1 Hz, beta isomer, total 1H, ratio 1:2, respectively), 2.28–1.10 (m, 13H), 0.86 (s, alpha isomer) and 0.71 (s, beta isomer, total 3H, ratio: 1:2, respectively). High-resolution mass spectrometric analysis ($C_{20}H_{26}O_4$ as the sodium adduct) Calculated MNa^+: 353.1729, observed MNa^+: 353.1727.

5.2.23. Synthesis of 3-hydroxy-2-methoxy-estra-1,3,5(10)-triene-17 α/β-carboxamide (23)

Steroid **22** (0.4 mmol) in freshly distilled $SOCl_2$ (2 mL) was stirred for 2 h at 70 °C, than evaporated. THF (2 mL) was added and NH_3(g) was bubbled through for 5 min at room temperature. The mixture was diluted with THF (2 mL) and solid byproduct was filtered off. The filtrate was evaporated and purified by chromatography (95:5:0.1 $CHCl_3$/MeOH/NH_4OH) to give compound **23**. ^{1}H NMR ($CDCl_3$) δ, 6.80 (s, 1H), 6.66 (s, 1H), 5.54–5.27 (m, 3H), 3.88 (s, beta isomer) and 3.87 (s, alpha isomer, total 3H, ratio 3:1, respectively), 2.85–2.74 (m, 2H), 2.43–1.22 (m, 14H), 0.92 (s, alpha isomer) and 0.79 (s, beta isomer, total 3H, ratio 1:3, respectively. High-resolution mass spectrometric analysis ($C_{20}H_{26}NO_3$) Calculated MH^+: 330.2069, observed MH^+: 330.2068.

References and notes

1. Pribluda, V. S.; Gubish, E. R.; LaVallee, T. M.; Treston, A. M.; Swartz, G. M.; Green, S. J. *Cancer Met. Rev.* **2000**, *19*, 173.
2. Zhu, B. T.; Conney, A. H. *Cancer Res.* **1998**, *58*, 2269.
3. Fotsis, T.; Zhang, Y.; Pepper, M. S.; Adlercrcutz, H.; Montesano, R.; Nawreth, P. P.; Schweigerer, L. *Nature* **1994**, *368*, 237.
4. Wilding, G.; Sweeney, C.; King, D. M.; Horvath, D.; Staab, M. J.; Fife, K.; Armstrong, V.; Soriano, J. V.; Pribluda, V.; Gubish, E. R.; Sidor, C. *Proc. Am. Soc. Clin. Oncol.* **2002**, *21*, #338.
5. Fogler, W. E. *Drug Disc. World* **2008**, *Fall*, 15.
6. Martucci, C. P. Metabolic Fate of Catecolestrogens. In *Catecol Estrogens*; Merriam, G. R., Lipsett, M. B., Eds.; Raven Press: NY, 1983; pp 115–122.

7. Adlercreutz, H.; Martin, F. *J. Steroid Biochem.* **1980**, *13*, 231; Adlercreutz, H. *N. Engl. J. Med.* **1974**, *290*, 1081.
8. (a) For data in humans: Sledge, G. W.; Miller, K. D.; Haney, L. G.; Nguyen, D. D.; Storniole, A. M.; Philips, E. N.; Pribluda, V. S.; Gubish, E. R. 38th Annual Meeting, American Society of Clinical Oncology, May 2002, Orlando, FL.; (b) Miller, K. D.; Murray, D. J.; Curry, E.; Haney, L. G.; McClaskey, G. W; Storniolo, A. M.; Philips, E.; Pribluda, V. S.; Gubish, E. R. 38th Annual Meeting, American Society of Clinical Oncology, May 2002, Orlando, FL.; (c) Fpr data in rats, Unpublished data, EntreMed, Inc.
9. Lakhani, N. J.; Sparreboom, A.; Xu, X.; Veenstra, T. D.; Venitz, J.; Dahut, W. L.; Figg, W. D. *J. Pharm. Sci.* **2007**, *96*, 1821.
10. (a) Cushman, M.; He, M.-H.; Katzenellenbogen, J. A.; Lin, C. M.; Hamel, E. *J. Med. Chem.* **1995**, *38*, 2041; (b) Cushman, M.; He, M.-H.; Katzenellenbogen, J. A.; Varma, R. K.; Hamel, E.; Lin, C. M.; Ram, S.; Sachdeva, Y. P. *J. Med. Chem.* **1997**, *40*, 2323; (c) Miller, T. A.; Bulman, A. L.; Thompson, C. D.; Garst, M. E.; Macdonald, T. L. *J. Med. Chem.* **1997**, *40*, 3836; (d) Miller, T. A.; Bulman, A. L.; Thompson, C. D.; Garst, M. E.; Macdonald, T. L. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1851; (e) Wang, Z.; Yang, D.; Mohanakrishnan, A. K.; Fanwick, P. E.; Nampoothiri, P.; Hamel, E.; Cushman, M. *J. Med. Chem.* **2000**, *43*, 2419; (f) Hughes, R. A.; Harris, T.; Altman, E.; McCallister, D.; Vlahos, R.; Robertson, A.; Cushman, M.; Wang, Z.; Stewart, A. G. *Mol. Pharmacol.* **2002**, *61*, 1053; (g) Cushman, M.; Mohanakrishnan, A. K.; Hollingshead, M.; Hamel, E. *J. Med. Chem.* **2002**, *45*, 4748; (h) Edsall, A. B.; Mohanakrishhnan, A. K.; Yang, D.; Fanwick, P. E.; Hamel, E.; Hanson, A. D.; Agoston, G. E.; Cushman, M. *J. Med. Chem.* **2004**, *47*, 5126; (i) Rao, P. N.; Cessac, J. W.; Tinley, T. L.; Mooberry, S. L. *Steroids* **2002**, *67*, 1079; (j) Agoston, G. E.; Shah, J. H.; LaVallee, T. M.; Zhan, X.; Pribluda, V. S.; Treston, A. M. *Bioorg. Med. Chem.* **2007**, *15*, 7524.
11. Shapiro, R. J.; Djerassi, C. *J. Org. Chem.* **1964**, *86*, 2825.
12. Peters, R. H.; Crowe, F. C.; Avery, M. A.; Chong, W. K. M.; Tanabe, M. *J. Med. Chem.* **1989**, *32*, 1642.
13. Schow, S. R.; McMorris, T. C. *J. Org. Chem.* **1979**, *44*, 3760.
14. Romanelli, M. G.; Becker, E. I. *Org. Syn. Coll. Vol. 5*; John Wiley and Sons, 1973. pp 552–554.
15. Fieser, L. F.; Jones, J. E. *Org. Syn. Coll. Vol. 3*; John Wiley and Sons, 1955. pp 590–591.
16. Shah, J. H.; Izenwasser, S.; Geter-Douglass, B.; Witkin, J. M.; Newman, A. H. *J. Med. Chem.* **1995**, *38*, 4284.
17. Carroll, F. I.; Gao, Y.; Abraham, P.; Lewin, A. H.; Lew, R.; Patel, A.; Boja, J. W.; Kuhar, M. J. *J. Med. Chem.* **1992**, *35*, 1813.
18. Kabalka, G. W.; Hutchins, R.; Natale, N. R.; Yang, D. T. C.; Broach, V. *Org. Syn. Coll. Vol. 6*; John Wiley and Sons, 1973. pp 293–298.
19. Berliner, D. L., Adams, N. W.; White, J. U.S. Patent 5783571, 1998.
20. Borch, R. F.; Bernstein, K. D.; Durst, D. H. *J. Am. Chem. Soc.* **1971**, *93*, 2897.
21. McGuire, M. A.; Sorenson, E.; Owing, F. W.; Resnick, M. F.; Baine, N. H. *J. Org. Chem.* **1994**, *59*, 6683.
22. LaVallee, T. M.; Zhan, X. H.; Herbstritt, C. J.; Kough, E. C.; Green, S. J.; Pribluda, V. S. *Cancer Res.* **2002**, *62*, 3691.
23. (a) Suwandi, L. S.; Agoston, G. E.; Shah, J. S.; Hanson, A. H.; Zhan, X. H.; LaVallee, T. M.; Pribluda, V.; Treston A. M., Bioorg. Med. Chem. Lett. in press.; (b) Agoston, G. E.; Shah, J. H.; Suwandi, L. S.; Hanson, A. D.; Zhan, X. H.; LaVallee, T. M.; Pribluda, V. S.; Treston, A. M., Bioorg. Med. Chem. Lett. in press.